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INTERPLAY BETWEEN POLYCYSTIN-1 AND EXTRACELLULAR MATRIX: INVESTIGATING THE REJ DOMAIN'S SIGNIFICANCE IN ADPKD PATHOGENESIS

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ABSTRACT

Polycystin 1 (PC1) plays a crucial role in the progression of autosomal dominant polycystic kidney disease (ADPKD), a potentially life-threatening monogenic disorder marked by the gradual formation of fluid-filled cysts that compromise renal function and may lead to end-stage renal disease. Polycystin 1 is a protein of structural complexity, featuring numerous domains and engaging in diverse functions such as cellular adhesion, signal transduction, and ion channel activity. Our goal was to unravel its interactions with other proteins and its involvement in the onset of cystic diseases, ADPKD. This exploration seeks to enhance our grasp of the intricate interplay between its structure and functions, offering valuable insights into potential therapeutic targets. Our focus in this study is to study and characterize a REJ domain located at the N-terminal extracellular region of PC-1 and study its interaction with various components of the extracellular matrix. By exploring these interactions, we can better understand their potential significance in both the healthy development of the kidneys and the underlying mechanisms of ADPKD. *In vitro*, pull-down assays were used to assess the binding of the REJ fusion to several ECM components. The results showed that the REJ fusion protein binds to collagen VI, integrin, and fibronectin. The addition of the REJ fusion protein to HEK293 embryonic kidney epithelial cells in culture resulted in a significant reduction in the rate of cell proliferation. These findings indicated that the REJ region serves as a mediator for the interaction between polycystin-1 and the ECM and highlights the functional role of polycystin-1 in cell-matrix and cell-cell interactions.

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Introduction

Autosomal dominant polycystic kidney disease (ADPKD) stands as the predominant genetic contributor to renal failure on a global scale. This ailment is characterized by its progressive nature, manifesting in cyst formation, kidney enlargement, and engagement of extrarenal organs like the liver, pancreas, spleen, and arachnoid membranes. It affects individuals across diverse racial backgrounds in adulthood, contributing to 6% to 10% of dialysis patients in the United States. Although cysts may be discernible in childhood or even before birth, the clinical symptoms typically emerge during the third or fourth decade of life [1].

ADPKD is characterized by the continuous development and enlargement of cysts in the kidneys, resulting in progressive kidney enlargement, extrarenal organ involvement (eg, liver, pancreas, spleen, and arachnoid membranes) and diminished quality of life [1]. It is caused by mutations in the PKD-1 or PKD-2 genes. In the ADPKD majority of cases (approximately 85%), the disease is caused by mutations in the PKD-1 gene located on chromosome 16p13.3. In the remaining cases, mutations in the PKD-2 gene are located on chromosome 4q21-q22. The therapy of ADPKD is directed towards reducing and slowing the progression of renal failure in ADPKD and includes control of hypertension and hyperlipidemia, dietary protein restriction, control of acidosis, and prevention of hyperphosphatemia [2]. Surgery to reduce the size of cysts can alleviate severe pain caused by cyst expansion in most cases, but not all. However, this surgical intervention is only a temporary solution and does not prevent ADPKD from progressing towards kidney failure [3].

ECM alterations have been suggested to be part of ADPKD. It was observed that the cellular basement membrane (BM) of cysts in human ADPKD kidneys in the end-stage had become thickened and extensively laminated, and there was abnormal regulation of gene expression of various components of the BM such as collagens, laminins, and proteoglycans by cyst epithelial cells. These changes in BM were also observed in dilated tubules and small cysts of early ADPKD kidneys [4].

The exact mechanisms that lead to the formation of cysts in ADPKD and subsequent kidney function loss are not yet fully understood [5]. For a better understanding, the relationship between PC-1 and ECM proteins should be clarified. Investigation of the interaction between a component of the ECM and PC-1, explains the initiation of cysts and the progression of ADPKD. The extracellular region of PC-1 has the potential to interact with ECM proteins, which are important in the signal transduction pathway, play a role in renal development and could be a very important step in molecular cystogenesis [6]. Evidence has shown that the disruption or loss of PC-1 activity inevitably results in subtle derangements of cellular calcium regulation, through several potential pathways, as the cellular calcium's abnormal homeostasis may subsequently alter the differentiation within affected cells [7]. Furthermore, it has been reported that the receptor of the egg jelly (REJ) domain is located in the N-

terminal extracellular region of the PC-1 protein, and may play a role in supporting the influx of calcium ions (Ca^{2+}) in the PC-1 domains [8].

The extracellular domain of PC-1 mediates cell-cell and cell-extracellular matrix binding, and it encompasses a multifunctional carboxy-terminal region [9].

REJ is a crucial part of the PC-1 ectodomain, covering approximately 1000 amino acids. This module contains numerous missense mutations responsible for various diseases. Nonetheless, there is limited knowledge about the configuration and role of this region [10]. The REJ proteins support Ca^{2+} influx, and the localization of the REJ protein domains in the extracellular region of the PC-1 protein allows it to interact with the ECM proteins and provides an excellent example model to investigate its function in ADPKD [11]. REJ localized near the GPS domain at the GPSG-protein-coupled proteolytic site, PC-1 undergoes cleavage, and this process involves the entire REJ region. GPS cleavage is an essential process for both kidney function and structure, and the number of mutations at the REJ region affects GPS cleavage by disruption [12].

Recent study has made a notable contribution to the understanding of the molecular mechanisms underlying inherited ADPKD by successfully expressing a REJ fusion protein in vitro., resulting in the production of a 13 kDa REJ protein that corresponds to the human REJ gene in exon 15. This novel research provides valuable insights into the molecular mechanisms involved in ADPKD and represents a significant advancement in this field [13].

Cells have a highly integrated system for proper tissue function via interaction with various types of extracellular matrix (ECM) proteins, which allow the exchange of signals between cells [14]). Basement membrane (BM) represents one type of ECM that is a crucial element in separating epithelial cells from the surrounding environment and connecting proteins in the ECM, it is composed of several proteins including laminin, collagen IV, heparan sulphate proteoglycans, integrin, fibronectin, and nidogen. The proteins in the ECM bind to receptors expressed on the BM through integrin and hemidesmosomes, which are cooperatively located on the cell surface [15]. The major and conserved components of the BM include laminins, type IV collagen (collagen IV), nidogens, and heparan-sulphate proteoglycans (HSPGs). Together, these components form a sheet that allows cells to adhere and maintain normal physiological functions. The BM also plays a crucial role in regulating many aspects of cell biology, such as cell polarity, proliferation, apoptosis, survival, migration, differentiation, and signaling [16].

Recent studies have shown that the alter in the ECM and BM composition are present in the early stages of cyst development in ADPKD; these changes include thickening and extensive lamination of cellular basement membranes, abnormal regulation of genes associated with collagens, laminins and proteoglycans, as well as overexpression of several integrins in cystic cells. The BM alterations were observed in dilated tubules and small cysts of early ADPKD kidneys, suggesting that changes in the ECM are early features of cyst formation [4]. Collagen IV is a crucial element of all BM and is widely distributed in BM structures [17]. Collagen IV is a crucial component of BM. It plays a critical role in tissue development, differentiation, maintenance, and remodeling [17]. Also, is implicated in the pathogenesis of several human disorders, including diabetic nephropathy, and angiopathy [18]. The main receptors that interact with collagen IV are integrins, which are a family of transmembrane glycoproteins that exist as αβ heterodimers. Integrins serve a variety of functions including adhesion, signaling, mechanotransduction, and regulation of cell growth and death. Among the many integrins expressed in epithelial cells, the α3β1 integrin specifically interacts with type IV collagen [19].

Collagen IV networks play a pivotal role in the architecture, signaling, and organization of the overall BM [20]. Type IV collagen plays a crucial role in both providing structural support and enabling signaling processes that are important for various physiological and pathological functions. Alport's syndrome, a chronic kidney disease, is typically caused by mutations in type IV collagen that have been extensively studied [21]. These effects are often mediated by specific integrins, particularly α1β1 and α2β1, which interact with the central triple helical domain of collagen IV protomer [22]. Also, type IV collagen is a unique type of collagen that is found exclusively in BM. It creates complex structures through interactions between different molecules, both within and between collagen molecules. These structures play an important role in regulating processes such as differentiation [17].

Integrins are receptors found on the surface of cells that consist of both alpha and beta subunits. They play a crucial role in the interaction between cells and the ECM by binding to various components of the ECM, such as fibronectin and collagen IV [23]. Multidomain adhesive proteins found in the ECM serve as ligands for a variety of integrins. Some integrins can bind to specific domains of multiple proteins, while certain adhesive proteins can bind to several different integrins. These interactions primarily facilitate adhesion between cells and the ECM, as well as between cells themselves [24]. Altered expression of integrin cell adhesion receptor subtypes has also been reported in ADPKD cells [25]. The investigation of the interactions between the extracellular domains of PC-1 and ECM components is crucial for understanding normal renal development and the pathogenesis of ADPKD. It has been demonstrated that isolated PC-1 domains, such as the C-type lectin and LRR, can bind to various ECM proteins located outside of cells [26].

Given the information provided, the primary objective of this study is to showcase the expression of a soluble REJ fusion protein and examine its impact on the proliferation of HEK 293 cells. Additionally, the investigation aims to explore the interaction of this protein with ECM proteins through the utilization of a pulldown assay. This step is considered essential in comprehending the protein's function and identifying relevant biological pathways.

Materials and Methods

Materials:

Most of the chemicals were purchased from Sigma Aldrich (UK). Human embryonic kidney (HEK 293) was kindly provided by Dr Baghdadi, Research Centre, King Faisal Specialist Center, Jeddah, Kingdom of Saudi Arabia. Dulbecco's modified eagle medium (DMEM), penicillin-streptomycin, trypan blue solution, trypsin-EDTA, phosphate-buffered saline (PBS) and dimethyl sulfoxide (DMSO), were purchased from Solar bio (China). The heat-inactivated fetal bovine serum (FBS) was obtained from Gibco Thermo Fisher Scientific (KSA). Thiazolyl blue tetrazolium bromide powder (MTT) was provided by Gold Biotechnology (USA).

Methods:

Cloning and Expression of the REJ domain as soluble protein

Cloning and expression of the MBP-REJ domain and fusion protein expression and purification with PureCube His-Affinity agarose were modified from the method described previously [13].

Human embryo kidney epithelial cell culture and proliferation assay
Cell Line and Passage:
HEK 293 cells at passage '9' were selected as the experimental model to investigate the impact of MBP-REJ fusion proteins on cellular proliferation.

Cultivation and Maintenance:
HEK 293 cells were maintained as monolayers in uncoated tissue culture flasks, with culture conducted using Dulbecco's Modified Eagle medium (DMEM). The culture medium was supplemented with 10% (v/v) fetal calf serum (FCS) and 1% (v/v) penicillin-streptomycin solution. The cells were cultivated in a controlled environment at 37°C with 5% (v/v) carbon dioxide (CO_2) and 95% air. The culture medium was refreshed every 2-3 days or as needed.

Subculturing:
Routine subculturing of cell lines was carried out at 37°C with 5% CO2. Upon achieving approximately 70% confluency, cell cultures were visually inspected under a phase-contrast microscope (Nikon, USA) to ensure optimal density and freedom from contamination. Subsequently, cells were detached using trypsin, and centrifuged at 1500 rpm for 10 minutes, and the resulting cell pellets were retained for cell counting using a hemocytometer.

Cell Seeding:
The cells were then seeded at a density of 5.0×10^4 cells/µL and allowed to adhere overnight before commencing the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) experiment.

Studying the effect of MBP-REJ on cell viability and proliferation:

MTT Cell Proliferation Assay
The influence of the MBP-REJ fusion protein on the growth of HEK293 cells was assessed using the MTT assay. This calorimetric assay gauges mitochondrial dehydrogenase activity in viable cells, as it converts pale yellow, soluble MTT into an insoluble purple formazan product. The intensity of the purple colour is directly proportional to the metabolic activity of the cells, with a deeper colour indicating higher absorbance values and, consequently, a greater number of viable cells [27]. HEK293 cells were cultured at a density of 5000 cells per 100µl in 96-well microtiter plates and incubated at 37°C overnight. Various concentrations of the MBP-REJ protein (0.5, 1, 1.5, and 2 mg/ml) were applied to the cells. The culture plates were then incubated at 37°C for 24, 48, and 72 hours. Control wells containing cells and medium were included. After 24 hours of incubation, 10 µl of MTT solution (3 mg/ml) was introduced into each well, followed by further incubation at 37°C. After a 4-hour incubation period, the supernatants were carefully removed. Subsequently, 100 µl of dimethyl sulfoxide (DMSO) was added to each well and thoroughly mixed to dissolve the formazan crystals.
The absorbance at 570 nm was measured using an enzyme-linked immunosorbent assay (ELISA) reader (BioTek Instruments, USA). The same procedure was repeated after 48 and 72 hr. The calculation of cell viability percentage was performed manually, following the formula established by Choudhury et al. in 2016 [28]. Changes in cell morphology were visually examined through a phase-contrast microscope (Nikon ECLIPSE ti-S, Japan) at 24, 48, and 72 hours after the treatment.

Interaction of MBP-REJ fusion protein with extracellular matrix protein
Pull-down assay
The possible interaction of a component of the extracellular matrix with polycystin-1 may be important in the initiation or progression of ADPKD [11]. A pull-down assay to study protein-ECM interaction was performed according to manufacturer instructions (Pull-Down Kit, Thermos Fisher, UAS).
HEK 293 cells were used in the pulldown assay. HEK 293 cells were grown as monolayers to confluency ($1x10^7$) on an uncoated tissue culture flask using a DMEME medium. The cells were washed 2X with ice-cold PBS. One milliliter of RIPA lysis buffer (150mM Nacl,1% Triton X100, 0.5% of Sodium Deoxycholate, 0.1 % SDS, 50 mM Tris, pH 8, Tris HCL, 20 mM Tris-HCL, pH7.5), phenylmethylsulfonyl fluoride Protease inhibitors PMFS (one tablet /10 ml RIPA buffer) was added (1 ml) to the cells and left on ice for 5 min. The cells were then scraped with a cell scraper (Costar Corning incorporated, USA) transferred to sterile 0.5 ml Eppendorf and kept on ice for 1h. During this period, the cell lysate was pipetted using small tips every 10 min to physically break down the cells. The disrupted cells were centrifuged down in a bench centrifuge at full speed (13000 rpm) for 30 min at 4°C. The supernatant was either used immediately or stored at –70 °C until further use.
Within our experimental methodology, we conducted optimization experiments involving various parameters, including different concentrations of MBP-REJ fusion protein, the volume of beads, incubation times, the number of washes, and the volume of cell lysate. The primary goal of these optimizations was to prevent any non-specific interactions between the protein and the His moiety or the beads.
To achieve this, we initiated the process by incubating 300 µl of purified MBP-REJ fusion protein with a 50% slurry of HisPur Cobalt Resin (100 µl) for 1 hour at 4 °C with gentle end-over mixing. Subsequently, the mixture underwent centrifugation in a bench centrifuge, running at 3341 rpm for 30 seconds to 1 minute. The resulting tube was labelled as the "bait flow-through" and was analyzed by running it on a 15% reduced polyacrylamide gel electrophoresis (SDS-PAGE). Additionally, the HisPur Cobalt Resin was subjected to 5 washes using a washing buffer composed of a 1:1 mixture of TBS and pull-down lysis buffer. To prepare the prey fraction, we started by adding 2.5 ml of Pierce Lysis Buffer (w/v) to the lysed cells. The tube was thoroughly mixed by repeatedly inverting it both upward and downward. Subsequently, the mixture was placed on ice for approximately 30 minutes with periodic inversions. Following this incubation, the mixture underwent centrifugation at 3341 rpm for 5 minutes to achieve clarification of the crude mammalian lysate. The resulting supernatant was carefully transferred

to a fresh tube and maintained on ice. Meanwhile, Imidazole (10 mM) was introduced to the remaining cell pellet. This particular tube was designated as the "mammalian prey lysate" and, once again, stored on ice.

In the experimental procedure, 800 µl of the prey protein was introduced into a microfuge tube and subsequently incubated at 4 °C for 2 hours on a rotating platform with gentle rocking. Following incubation, the samples underwent centrifugation at 3341 rpm for 1 minute, and the resulting tube was designated as the "prey flow-through" and placed on ice. Subsequently, the supernatants were gathered for analysis using a 15% reduced SDS-PAGE based on gel percentage. The protein-capturing beads were subjected to washing with 1 ml of ice-cold lysis buffer, which had initially been employed to lyse the cells. These tubes were then centrifuged at maximum speed (1500 rpm) for 1 minute. The washing step was iterated 3 to 4 times. Elution Buffer (250 µl) was added to the microfuge tube, and incubated for 5 min with gentle rocking on a rotating platform. The sample was centrifuged at 3341 rpm for 1 min. This tube was labelled as “Elution 1” and it was placed on ice. The elution steps were repeated 5x.

The experiment to pull down proteins was optimized by changing the concentration of the MBP-REJ fusion protein, the number of beads used, incubation times, the number of washes, and incubation periods. Samples, including non-treated His-Pur Cobalt Resin, purified bait, cells lysate, bait flow through, prey flow through, and bait-prey elution, were electrophoresed by using 15% reduced SDS-PAGE.

Characterization of protein-protein interaction by mass spectrometry

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) (Ultra-flex III Manufacturers by Bruker Corporation, USA) was used to identify the proteins that have been pulled down with MBP-REJ, which has been subjected to SDS-PAGE prior to characterization. The relevant stained protein bands were excised from the gel and stored in 0.1% acetic acid and the resulting proteins were analyzed by MALDI-TOF MS which was followed by database searching (Swiss Prot) [29]. The analysis process was carried out in cooperation with King Abdullah University of Science and Technology KAUST, Jeddah, Saudi Arabia).

Statistical analysis

The data obtained from the study were subjected to analysis using GraphPad Prism version 8.2.1, also known as Prism 8. To determine if there were any significant differences between the groups that received treatment and the control group, a statistical test called one-way ANOVA was employed, specifically utilizing Dunnett's multiple comparisons tests. The results were presented as the mean value accompanied by the standard deviation (SD). Statistical significance was considered when the p-value was less than 0.05 ($P < 0.05$).

Results

Studying the effect of MBP-REJ on cell viability and proliferation

The effect of the MBP-REJ fusion protein on the cellular proliferation of the HEK 293 cell line was evaluated by MTT assay. The purified MBP-REJ fusion protein (0.5, 1, 1.5 and 2 mg/ml) was added to the multi-well plates containing MBP-REJ fusion protein. The number of viable cells in culture after treatment with MBP-REJ fusion protein over time was monitored.

The initial cell density was set at 5000 cells per well. Following a 24-hour incubation period, a marginal decrease in viable cell numbers in multi-well plates was observed alongside an escalation in the MBP-REJ fusion protein compared to untreated cells. The mean± SD of the control group was 0.322± 0.012, with a corresponding 100% viability calculated from the control equation. With increasing concentrations from 0.5 to 2 mg/ml, the mean± SD exhibited a decline to 0.276± 0.048, 0.255± 0.051, 0.249± 0.004, and 0.232± 0.039 µg/ml, respectively. This decrease in mean± SD reflected a reduction in the percentage of viable cells in comparison to the control. The percentage of cell viability concurrently diminished to 85.67%, 79.13%, 77.26%, and 71.96%, respectively, indicating an inverse correlation between cell cytotoxicity and concentration. As the concentration of the fusion protein increased, cell cytotoxicity also increased, reaching 14.33%, 20.87%, 22.74%, and 28.04%, respectively (Table 1). Notably, at a concentration of 2 mg/ml of fusion protein, a significant difference in the mean± SD was observed, with a p-value of 0.014.

As the incubation period extended to 48 hours, there was an augmentation in the number of viable cells in the control group, while a noticeable decrease in viable cells became apparent in the treated wells. The initial mean± SD of the control was 0.283± 0.049, with a corresponding 100% viability. For treated cells with concentrations ranging from 0.5 to 2 mg/ml, the mean± SD showed a decline to 0.280± 0.054, 0.261± 0.046, 0.250± 0.059, and 0.241± 0.042 µg/ml, respectively. Simultaneously, the percentage of viable cells dropped from 100% in untreated cells to 99.12%, 92.22%, 88.22%, and 85.33% for treated cells. Correspondingly, cell cytotoxicity increased from 0.88% at a concentration of 0.5 mg/ml to 7.78%, 11.78%, and 14.67% with escalating concentrations (Table 1). Notably, there were no significant differences in the mean± SD between 0.5 and 1 µg/ml compared to the control. However, significant differences were observed in the mean± SD at concentrations of 1.5 and 2 mg/ml when compared to the control. As the incubation period extended to 72 hours, there was a decrease in the number of viable cells in the control group. The mean± SD of the control was 0.280± 0.055, with 100% viable cells. In the treated wells, there was no significant reduction in the number of viable cells. The mean± SD for concentrations of 0.5-2 mg/ml were 0.270± 0.041, 0.258± 0.091, 0.214± 0.014, and 0.202± 0.006. A reverse relationship between concentration and cell viability was observed, with the percentage of viable cells decreasing from 100% in untreated cells to 96.3%, 92.04%, 77.88%, and 72.17% in treated cells with concentrations of 0.5-2 mg/ml. Additionally, an inverse relationship was noted between cell cytotoxicity and concentration, with cytotoxicity increasing to 3.7%, 7.96%, 22.12%, and 27.83%, respectively (Table 1).

Table (1): Evaluation of cell viability using MTT Assay for control and various Concentrations of MBP-GPS Fusion Protein

Groups	**mean± SD**	**C (%)**	**Cell Cytotoxicity (%)**	***P* value**
24 hours				
Control	0.322± 0.012	100	0	
(0.5 mg/ml)	0.276± 0.048	85.67	14.33	0.414

(1 mg/ml)	0.255± 0.051	79.13	20.87	0.100
(1.5 mg/ml)	0.249± 0.004	77.26	22.74	0.060
(2mg/ml)	0.232± 0.039	71.96	28.04	**0.014***
48 h				
Control	0.283± 0.049	100	0	
(0.5 mg/ml)	0.280± 0.054	99.12	0.88	1.000
(1 mg/ml)	0.261± 0.046	92.22	7.78	0.982
(1.5 mg/ml)	0.250± 0.059	88.22	11.78	0.902
(2 mg/ml)	0.241± 0.042	85.33	14.67	0.789
72 h				
Control	0.280± 0.055	100	0	
(0.5 mg/ml)	0.270± 0.041	96.3	3.7	1.000
(1 mg/ml)	0.258± 0.091	92.04	7.96	0.785
(1.5 mg/ml)	0.214± 0.014	77.88	22.12	0.690
(2 mg/ml)	0.202± 0.006	72.17	27.83	0.430

Data are expressed as mean±SD, C%: cell viability, SD: standard deviation, *: P <0.05

Interaction of MBP-REJ fusion protein with ECM proteins

Pull-Down Assay

The HEK 293 cells used in the pull-down assay are presented in Figure 1. The Figure demonstrates different seeding numbers for HEK 293 cells. The pull-down assay studied the possible interactions between MBP-REJ and ECM proteins.

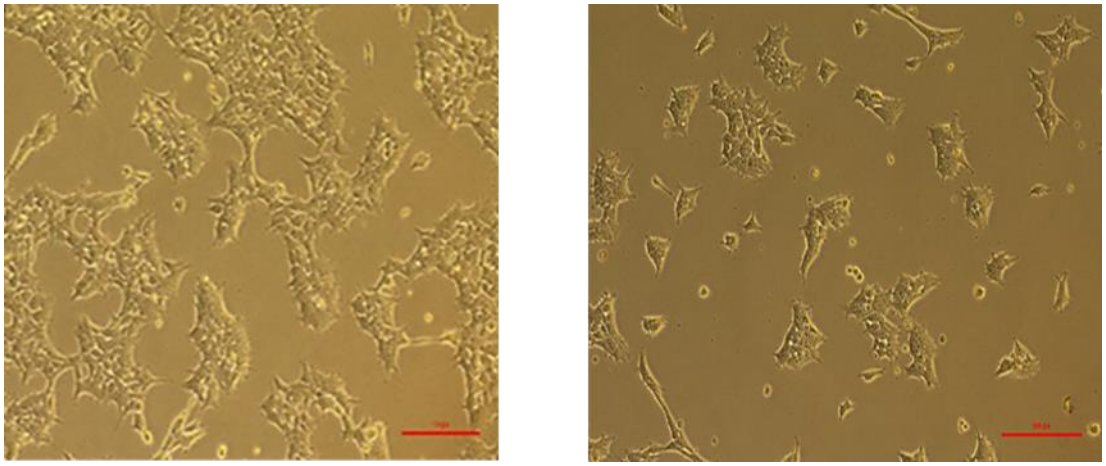

Figure 1: The HEK293 monolayer cells line in different seeding numbers. Scale bar, 100 µm.

The pull-down experiment was analysed via 15% SDS-PAGE. In Figure 2, lane 1 shows the HEK 293 cell lysate, while lanes 2, 4, 7, 8 and 9 serve as negative controls with untreated resin. In lanes 3 and 6, the bait protein MBP-REJ fusion is visible as a narrow band that has a size of 53 kDa. Lanes 5 and 10 show the prey proteins that interacted with MBP-REJ, and a protein band of approximately 100-127 kDa was detected in those lanes.

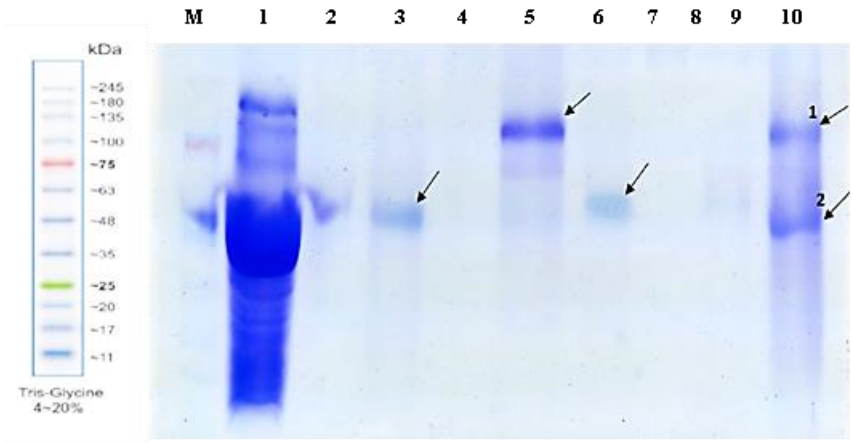

Figure (2): This figure represents the pull-down experiment. The electrophoresis was carried out in 15% SDS-PAGE. Lane 1, HEK 293-cell lysate. Lanes 2, 4, 7, 8 and 9 are untreated resins as a control. Lanes 3 and 6 are the MBP-REJ fusion protein (bait) (~53 kDa as the arrow indicated). Lane 5 was prey flow through. Lane 10 is the bait-prey elution that has two protein bands prey (~ 100-127 kDa as arrow 1 indicated), and MBP-REJ fusion protein (bait) (~53 kDa as arrow 2 indicated). Lane M is the molecular weight protein marker.

Characterization of protein-protein interaction by mass spectrometry

MALDI-TOF MS was employed to analyze the bands in lane 10 of Figure 2. The proteins identified in the analysis of the protein bands in lane 10 were recorded in Table 4.1. The UniProt database was then utilized to determine the protein masses associated with the MBP-REJ fusion protein.

Several candidates' proteins were identified in band lane 10, Figure. 2. Four proteins were pulled down using MBP-REJ fusion protein (Figure 2).

The potential for detecting a protein mixture within the gel band is possible. The proteins identified in lane 10, Figure 2, exhibited estimated molecular weights of approximately 100 and 127 kDa (Table 2). Table 2 includes information such as the predicted molecular weight, isoelectric point (pI), protein name, and mass for each identified protein. Among the potential protein candidates suggested by UniProt and ExPASy were two types of collagen chains, with estimated molecular weights of 100 kDa and 108 kDa, an integrin protein with a molecular weight of 127 kDa, and a Fibronectin protein with a molecular weight of 120 kDa. All four proteins were identified within a single, large, transparent band.

Table (2): ECM proteins that interacted with MBP-REJ fusion protein detected by MALDI-TOF MS using a pull-down assay

Protein name	UNI port Accession #	MW	PI
Collagen alpha 1(VI) chain	A0A087X0S5_HUMAN	100	5.89
Collagen alpha 1(IV) chain	P53420.CO4A4_HUMAN	108	5.20
Integrin alpha	A0A087X131-HUMAN	127	6.19
Fibronectin	H0Y4K8_HUMAN	120	5.52

Discussion

ADPKD is the prevailing genetic disorder that poses a significant threat to individuals' lives and is responsible for approximately 50 per cent of cases resulting in ESKD during the fifth and sixth decades of life (Krappitz *et al*., 2023 [30]). High frequencies of mutations that may lead to an early onset of a severe form of ADPKD were reported in the Saudi population [31, 32].

Since the identification of the *PKD-1* and *PKD-2* genes, there has been a surge in research interest in PKD over the past three decades [33]. ADPKD poses both clinical and health challenges, emphasizing the need for continued research and intervention in this field.

Our comprehension of the genes responsible for ADPKD has advanced as a result of the progress made in genetics [34]. For this purpose, our study was designed to investigate and characterize the structure and function of PC-1 and study the molecular mechanism by which the REJ domain of PC-1 interacts with ECM proteins to raise its potential importance in the aetiology of ADPKD. This study represents a pioneering effort in exploring the functions and mechanisms of previously unknown REJ proteins to understand their structure and function. The functionality of the protein is not compromised by the presence of the MBP tags. This was demonstrated in a study by Guo et al. (2018) [35], in which they successfully produced bioactive leukaemia inhibitory factor (LIF) by fusing it with MBP, resulting in the formation of the MBP-LIF fusion protein. They utilized the *E. coli* system for protein expression and employed a one-step purification method using gravitational affinity chromatography. The purified MBP-LIF products demonstrated the ability to selectively inhibit the growth of M1 cells, and this inhibition showed a dose-dependent pattern. Importantly, their findings indicated that the presence of the MBP tag in the MBP-LIF fusion protein did not hinder the bioactivity of mLIF [35].

HEK 293 is a commonly used human cell line in scientific research because it offers several significant benefits. One key advantage is its ability to undergo post-translational modifications that closely resemble those found in human cells. HEK 293 is preferred due to its high efficiency in accepting foreign genetic material (transfectivity), its rapid proliferation rate, and its capability to grow in a culture medium without the need for serum in a suspension environment [36, 37]). To support our hypothesis, we employed the MTT assay to investigate the impact of the MBP-REJ fusion protein on the growth of HEK 293 cells expressing normal PC-1. This assay enabled us to assess cell viability by measuring the number of viable cells in culture following treatment with the fusion protein, both in a dose-dependent and time-dependent manner. Our results demonstrated a significant reduction in cell proliferation, which aligns with previous findings [3]. They reported various changes in ADPKD cyst-lining epithelial cells, including alterations in proliferation, accumulation of fluid, remodelling of the ECM, and elevated expression of collagen and integrins. This convergence between our observations and the findings reported by Zhang et al. (2020) [4], and Grantham. (1992) [3], underscores the validity of our hypothesis and highlights the potential implications of the MBP-REJ fusion protein in modulating cell growth and ECM dynamics in the context of polycystic kidney disease.

This study investigated the effects of treating HEK293 cells with MBP-REJ fusion protein over 24 hours. We observed a slight reduction in the number of viable cells in the treated wells compared to the control group, as the concentration of MBP-REJ fusion protein increased. This decrease in cell viability was, with higher concentrations leading to a greater reduction. However, the differences in cell viability between the concentrations and the control group were not statistically significant except for the concentration of 2 mg/ml of the MBP-REJ fusion protein. This suggests that at this early time point, the fusion protein had a limited impact on cell proliferation. When the incubation period was extended to 48 and 72 hours, the reduction in the number of viable cells in the treated wells was not significant. The control group showed an increase in cell viability, while the treated cells exhibited a further decrease in viability with increasing concentrations of the fusion protein. Statistical analysis revealed that the mean viability of cells treated with 2 mg/ml concentrations was significantly different from the control group. These findings indicate that the MBP-REJ fusion protein has a cytotoxic effect on HEK 293 cells after hours of exposure.

The impact of certain proteins on the cytotoxicity of the HEK-293 cell line used to be assessed using the MTT assay. These proteins hold significant importance in various scientific domains, including environmental studies, medicine, and pharmaceutical research. The investigation of their cytotoxic effects is crucial for gaining insights into their potential

implications and applications in these fields [38, 39, 40, 41]. It has been reported that the disruption of PC-1 function is associated with increased proliferation in ADPKD. Therefore, the observed reduction in proliferation in our study could be attributed to the interference of the MBP-REJ fusion protein with crucial cellular processes involved in cell survival, proliferation, or metabolism, potentially leading to impaired cell growth and eventual cell death [42, 43, 44]. In a study conducted by Perumal et al. (2019) [45], the MTT assay was utilized to investigate the effects of Tannic acid on normal HEK293 cells. The objective of their study was to explore the antiproliferative and antioxidant effects induced by Tannic acid in human embryonic kidney cells. The MTT assay results demonstrated that higher concentrations of Tannic acid led to an increase in both cell viability and the rate of cellular proliferation. In summary, the findings from our study, along with other research [42, 43, 44, 45] provided evidence for the impact of PC-1 disruption on cell proliferation and highlighted the potential of the MBP-REJ fusion protein to influence cellular processes related to growth and viability.

The role of PC-1 in the regulation of Ca^{2+} oscillations and the molecular mechanism elucidating the connection between disrupted Ca^{2+} homeostasis and heightened cell proliferation in ADPKD were investigated. Depleting the naturally occurring PC-1 protein in HEK293 cells resulted in intensified Ca^{2+} oscillations upon serum stimulation, subsequently activating the nuclear factor of activated T cells and promoting progression through the cell cycle. Similarly, kidney cystic cell lines with PC-1 mutations displayed increased Ca^{2+} oscillations and enhanced cell proliferation. However, these abnormal characteristics were alleviated in cells expressing exogenous PC-1 [46]. Our recorded suppressing effect of a specific dose of MBP-REJ fusion protein on cell proliferation might be interpreted in two different ways. One of them is completely close to our hypothesis and consistent with Vasileva et al. (2021) [47], and Torres and Harris. (2009) [48], that REJ of PC-1 may act as a receptor that regulates cell division by switching on/off the cell signalling upon its binding to cell membrane receptors such as integrin; since the REJ domain is close to the cell membrane. Furthermore, the localization of PC-1 and -2 on the surface of the primary cilium in a normal kidney, acts as mechanoreceptors that regulate calcium entry, which in turn stimulates various intracellular pathways to inhibit cell proliferation [49, 50] (Figure 3).

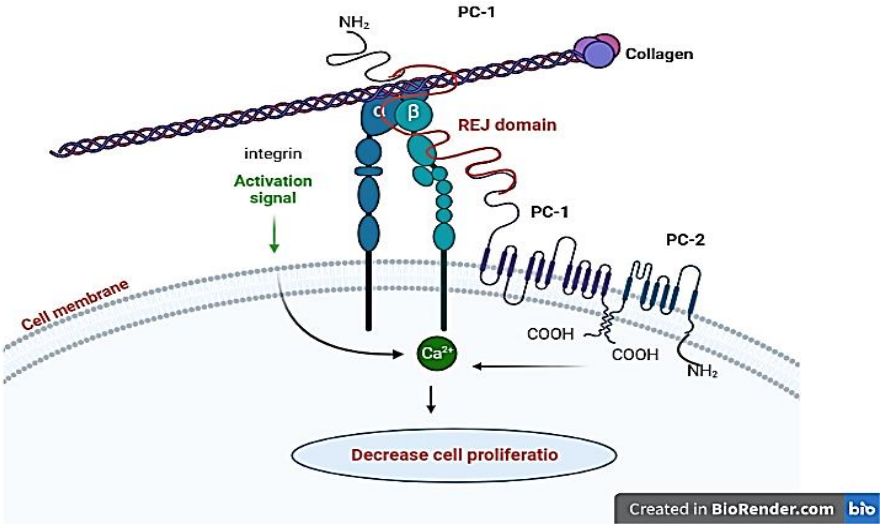

Figure 3: Our predicted model suggests that the localization of the REJ domain close to the cell membrane regulates Ca^{2+} influx and suppresses cell proliferation.

Another possible explanation was provided by Malhas et al., (2002) [6] suggesting that the decrease in proliferation could be attributed to the competition between the GST-LRR fusion protein and LRR-PC-1 in binding to the surface receptor collagen and its receptor integrin. This competition triggers a signalling pathway that inhibits further cell division [6]. In our study, we propose a similar mechanism, where MBP-REJ might also compete with REJ-PC-1, leading to a reduction in cell proliferation (Figure 4).

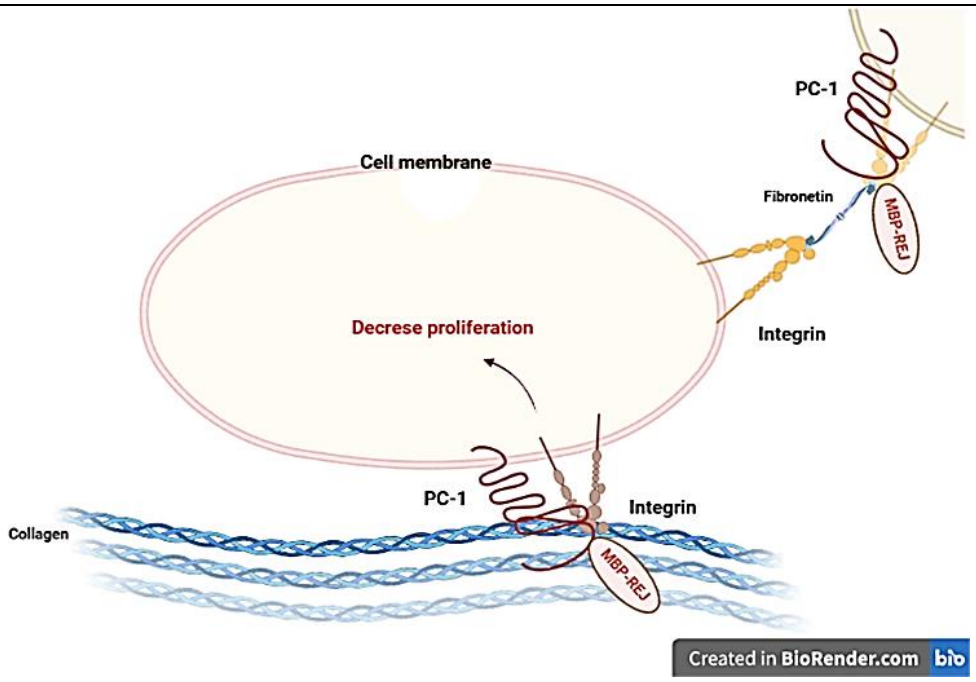

Figure 4: Predicated model to explaining the competition of MBP-REJ fusion protein and REJ of policystin-1 on binding to a surface receptor collagen and its receptor integrin, to decrease cell proliferation.

In vitro, model where cells were transfected with a mutated REJ domain, leading to the formation of cyst-like structures with a spherical shape. This outcome strongly emphasizes the significant contribution of the REJ motif in the development of ADPKD. The result underscores the crucial role of the PC-1 protein in maintaining the normal functioning of the kidneys. Additionally, they highlight that the N-terminal domain of PC-1, which encompasses the REJ domain, is particularly susceptible to various mutations that play a role in the development of ADPKD [51].

The REJ domain of PC-1, which effectively interacted with various ECM components including collagen IV, integrin, and fibronectin may initiate a cascade of signal transduction pathways involved in normal cell development. Ultimately, the effect of REJ fusion protein on HEK 293 cell proliferation emphasized our proposed molecular mechanism. The primary objective of our study was to examine the interaction between the REJ domain of PC-1 and ECM basement membrane proteins, aiming to propose a potential molecular mechanism underlying the development of ADPKD. To achieve this, we employed a novel approach known as the His pull-down technique, which was followed by data analysis using MALDI-TOF MS. The protein identification was conducted using MALDI-TOF-MS, and the corresponding accession number in the UniProt database was utilized for the identification process [5, 52, 53]. This method is consistent with our chosen approach, further reinforcing the validity of our findings. MALDI is particularly advantageous for the swift identification of proteins that have been isolated through gel electrophoresis. The streamlined sample preparation procedure associated with MALDI makes it more efficient and less time-consuming compared to other methods. Also, MALDI exhibits higher sensitivity, enabling the detection of lower abundance proteins with greater accuracy. As a result, MALDI is a well-suited approach for the rapid and reliable identification of proteins separated via gel electrophoresis [52]. In contrast, Han et al. (2022) [54] demonstrated that the combination of pull-down assays with liquid chromatography-tandem mass spectrometry (LC-MS/MS) allowed for the identification of protein interactions.

In a study performed by Paramasivam et al. (2021) [55], the focus was on investigating the role of Mitochondrial ribosomal small subunit (MRPS) proteins in tumorigenesis. To gain insights into the functions of these proteins, the researchers employed a valuable approach of mapping protein-protein interactions (PPIs) onto well-known cellular processes. This strategy allowed for the identification of novel protein functions. To facilitate their investigations, the researchers generated fusion proteins of GST (Glutathione S-Transferase) and MRPS. The successful generation of GST/MRPS fusion proteins was confirmed through MALDI-TOF analysis. The methodology employed in this study aligns with the conducted pull-down assays, as described by Paramasivam et al., using HEK293 cell lysate as a source of anchor proteins. Subsequently, a nano-liquid chromatography/tandem mass spectrometry (nLC/MS/MS) analysis was carried out to identify and analyze the protein interactions associated with MRPS. This comprehensive approach, utilizing mass spectrometry techniques, offered valuable insights into the protein-protein interactions involving MRPS and their potential roles in tumorigenesis [55]. Our results were in concordance with the findings of this study.

Other studies, observed a similar phenomenon, documenting the interaction between the PC-1 C-type lectin domain and various ECM proteins, such as collagen types I, II, and IV, in an in vitro setting. They also identified an interaction between unidentified components of cyst fluid from ADPKD patients and the PC-1 C-type lectin domain, suggesting its involvement in cell-matrix interactions [26]. Additionally, our findings revealed the binding of several basement membrane proteins, ranging from 100 to 127 kDa, to the MBP-REJ fusion protein, including collagen alpha1(VI) chain, fibronectin, and integrin alpha. The presence of a diverse protein population may explain the identification of various proteins in the present study.

As the significance of the ECM composition, organization, and stiffness for maintaining tissue equilibrium becomes more evident, there is a growing demand to unravel the intricacies of how cells perceive, construct, and modify the ECM during dynamic tissue remodelling processes. Depending on some studies recorded the integrin role as transmembrane adhesion receptors in binding to collagen IV. Since, cells do interact directly with collagens via the four collagen-binding integrins α1β1, α2β1, α10β1, and α11β1 [19, 56]. Therefore, our proposed mechanism might be that upon binding of the MBP-REJ

fusion protein with collagen, it interferes with downstream signalling pathways, particularly that involving integrin cascade, which is important for cell proliferation and migration. This interference could decrease cell proliferation in ADPKD, potentially slowing down the progression of cyst formation. Also, the REJ may trigger a suppressant effect on cell proliferation by binding to a putative cell surface receptor (integrin) (Figure 5).

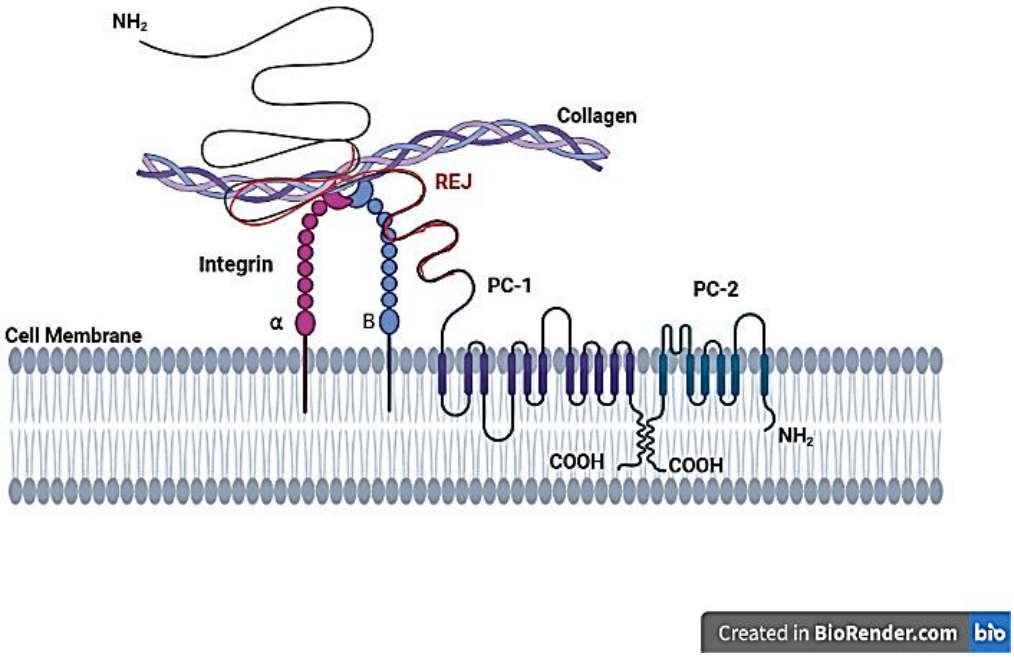

Figure (5): The interaction between MBP-REJ and ECM proteins is predicted by our model

Our study represents novel research that significantly contributes to the understanding of the molecular mechanisms underlying inherited ADPKD diseases. The REJ domain of PC-1 exhibited effective interactions with various ECM components, including collagen IV, integrin, and fibronectin, suggesting its involvement in initiating signal transduction pathways crucial for normal cell development. Additionally, the impact of the REJ fusion protein on HEK 293 cell proliferation further supports our proposed molecular mechanism.

Conclusion

In this study, the effect of REJ fusion protein on HEK 293 cell proliferation was presented. The Pull-down assay results were supported with MALDI-TOF MS data that proved the interaction between REJ fusion protein and ECM proteins including collagen, integrin, and fibronectin. The addition of REJ fusion protein to the HEK 293 cells caused a decrease in the cell's proliferation that may lead to the use of the REJ protein as a targeted therapy for ADPKD disease. Our results demonstrated a powerful experimental approach to further studying the function, and REJ- ECM interactions of proteins and should pave the way to systematically characterizing the effects of disease-causing mutations in the REJ module of human PC-1.
Further studies are recommended to define the structural/functional relationship between PC-1, integrin, and collagen in renal development, polycystin-deficient organisms, and ADPKD cells. Further investigations are required to elucidate the underlying mechanisms and understand the specific interactions of the REJ fusion protein with cellular components. These findings will provide valuable insights into the potential utility of the fusion protein for further investigations and potential therapeutic applications. Developing targeted therapeutic interventions is crucial to understanding the mechanisms involved in mechano-transduction and the role of polycystin in the proliferation associated with ADPKD. Additional research is necessary to explore these mechanisms in more detail and identify potential strategies to modulate cellular proliferation, ultimately aiming to slow down the progression of ADPKD.

Conflict of interest:
The authors have no conflicts of interest to declare.

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Not applicable.

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